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Clinco-Pathological and Biochemical Studies on Sheep Fed Aflatoxin Contaminated Ration

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Fifty sheep from Suez Governorate were suffered from high mortality rate (10% in the herd), weakness, depression, congestion of all mucous membrane and intestinal diarrhea. Ration analysis revealed presence of aflatoxin B_1 , contamination (300 ppb). Hemogram, biochemical and histopathological examination demonstrated. Hemogram in aflatoxicated sheep showed significant decrease in red blood corpuscles (RBCs) count, Hemoglobin % and pack cell volume (PVC). Normocytic norm chronic anemia as well as leucopenia as compared to control values. Hypoprotenemia, hypoalbumin, elevated liver and kidney enzyme were observed. Also, there was decrease in serum electrolyte (Ca, P, Mg, Na, K) as result of decrease re-absorption from the inflamed renal tubules. Also, demonstrated high residue of aflatoxin in liver, kidneys and muscles of affected sheep compared with normal one. Pathological examination of internal organs revealed severe damage in lung, heart, liver, kidney and intestine, that aflatoxins induced severe hepatic, renal and cardiac lesions which cause mortalities.

Aflatoxins are a group of structurally similar difurano coumarin elaborated as secondary metabolites produced by genus *Asperigillus* and are considered as most potent naturally occurring carcinogens (Kaaya *et al.,* 2006; Mohamed and Metwally, 2009; Prabakaran and Dhanapal, 2009). Peraica *et al.,* 1999 reported that aflatoxins are acutely toxic, immunosuppressive, mutagenic, teratogenic and carcinogenic compounds. Also, the main target organ for toxicity and carcinogenicity is the liver.

When livestock eat aflatoxin- contaminated feed, it causes many health problems Aflatoxin B1 (AFB_1) acts as a hepatotoxicant, hepatocarcinogen and mutagen (Alwakeel, 2009). The acute toxic effects of AFB_1 include hemorrhaging and death. Chronic exposure of aflatoxin affects growth rate, feed efficiency and susceptibility towords the bacterial and viral diseases (Yousef *et al.,* 2003). Lipid peroxidation and oxidative DNA damage are the principal manifestation of AFB1- induced toxicity which could be mitigated by antioxidants (Patel and Sail, 2006). Iowa State University (2011) recorded that small amounts cause mild or negligiable effects and large amounts cause serious effects. Animal most resistant are fedlot beef cattle, open cows and sheep. Aflatoxin is excreted rapidly from the body, so detectable levels may be gone within a few days to one to two weeks.

The aim of the present work was to investigate the etiological agents of mortalities in sheep herd on selected hematological, biochemical and histopathological response of sheep.

Material and methods

Fifty sheep of different age from herd consisted of 2000 sheep of which 200 (10% of the herd) were died within two weeks in Suez governorate. Bacteriological virological, mycological and parasitological examinations were carried out in (AHRI). Water, ration, fecal samples and rumen contents were collected, in addition two blood samples were collected to determine hemogram and some biochemical parameters. Postmortem examination was done immediately after slaughter and tissue specimens from internal organs were taken to histopathological examination.

Rations. Constituents were analyzed for determination of crud protein, fat, fiber Calcium, Phosphorus, Magnesium, Sodium and potassium by Oser (1979). Two ration samples were collected for analysis, one from mangers in front of animals and the other from the stored stock. These samples were used for determination of aflatoxin by thin layer chromatography as described by AOAC (1995).

Blood sampling. Blood samples were collected by the Jugular vein puncture from each animal. The first blood sample was anticoagulated with dipotassium ethylene-diamine-tetra acetic acid (EDTA) and used for determination of erythrocytic count (RBCs), hemoglobin concentration (Hb %), packed cell volume (PCV), total and differential leucocytic count (WBCs), in addition to values of red blood

indices including mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were determination according to Feldman *et al.,* (2000).

Blood sample was collected without anticoagulant for clear serum separation to be used for estimation of the activity of alanin-aminotransferase (ALT) and Aspartate-aminotransferase (AST) according to Reitman and Frankel (1957). Serum alkaline phosphatase was determined according to the modified methods of Kind and King, (1954). Serum total proteins were analyzed using the method of Henry *et al.,* (1974) and albumin according to Doumas *et al.,* (1971). Globulins were determined by difference between TP and albumin. Concentrations of urea and creatinine were determined according to Patton Crouch (1977) and Husdan and Rapoport, (1968) respectively. Colourometric determination was done for calcium and phosphorus according to Gindler and King (1972) and Goldenberg (1966) respectively.

Tissue specimens. Postmortem examination was done immediately after death and tissue specimens from lung, heart, liver, kidney and intestine were collected and fixed in 10% neutral buffer formalin. They were routinely processed by standard paraffin embedding technique, sectioned at 4µ and stained with Hematoxylin and Eosin (Bancroft and Gamble, 2008).

Statistical analysis. The obtained data were analyzed by using t-student test according to SPSS 14 (2006).

Results

Clinical signs and Mortality rate. Diseased animals in the herd suffered from dullness, weakness with started to lose their normal vitality. Mortality in the herd was investigated within 2 weeks. Mortality rate reached to 10% of the herd.

Postmortem findings. The dead sheep showed hydrothorax and sub-pericardial petechial hemorrhages. the abdominal cavity showed ascites with the presence of petechial hemorrhage which scattered on liver and kidney. The gall bladder distended and engorged with bile. Intestine showed severe congestion on the serosal and mucosal surfaces.

Laboratory investigation

Analysis of ration. Table (1) shows the composition of ration collected from healthy and affected sheep.

Hematological studies. Tables (2 and 3) demonstrated changes in the hemogram in aflatoxicated sheep. The erythrogram showed significant decrease in RBCs count, Hb and PCV (normocytic normochromic anemia). As compared with control values, leucogram demonstrated significant leucopenia as compared to control value.

Serum biochemical analysis. The values of the serum samples investigated are given in table (4). High significant decrease was recorded in serum total proteins, albumin and globulins. A significant increase in serum ALT, AST, GGT, AP, Urea and creatinine were also recorded. Table (5) present significant decrease in serum calcium, inorganic phosphorus, magnesium, potassium, sodium, Vit. A and Vit. E concentrations. Result in table (6) revealed high residue of aflatoxin B_1 in liver, kidneys and muscles when compared with normal ones.

Histopathological examination

Liver. Some hepatocytes suffered from necrosis, while most of them showed vacuolar degenerative changes. Edema dispersed the hepatocytes and disorganization of liver cell arrangement were noticed (Fig. 1).

Hemorrhages were observed in between the hepatocytes. Portal area showed newly formed bile ductules. Moreover some vascular cells with nuclear division were scattered in the portal area. The portal blood vessels were congested (Fig. 2).

Kidney. Periglomerular edema as well as severe edema dispersed the renal tubules were noticed. Some cells lining the renal tubules were suffered from vacuolar degenerative changes and others were necrosed (Fig.3). In addition to interstitial hemorrhage were observed (Fig.4).

Heart. Most of cardiac muscles suffered from zenker necrosis. Moreover, severe edema dispersed the cardiac muscles which admixed with mononuclear inflammatory cells (Fig. 5) Hemorrhage were noticed in between cardiac muscles (Fig. 6).

Lung. Some bronchi were dilated with proliferation of lining epithelial cells. Mild emphysema of some pulmonary alveoli were evident in many instances. Some capillaries of alveolar septa were congested, in association with hemolysed blood (Fig. 7 and 8).

Intestine. Hemorrhages showed in-between the intestinal glands and in the villi as well as infiltration of mononuclear inflammatory cells, also complete necrosis of some intestinal glands were seen (Fig. 9). Some cases showed infiltration of oesinophils (Fig.10).

Table (1): composition of ration analysis %.

Parameter	Contaminated ration	Uncontaminated ration
Humidity %	8	7.8
Ash %	9	10
Crud protein %	14	13
Ether extract %	4.3	4.1
Calcium mg/100gm	2.1	2
Inorganic phosphorus mg/100gm	1.3	1.2
Magnesium (mg/100gm)	2	1.9
Potassium (mg/100gm)	95	100
Sodium (mg/100gm)	100	105
Aflatoxin B1 (ppb)	300	Nil
Ochratoxin (ppb)	Nil	Nil

PPb (part per million) mg /100gm = mill gram/ 100 gram

Table (2): Mean values ± SE of erythrogram of aflatoxicated sheep.

Group	RBCs ($X10^6$ /µl)	Hb (g/dl)	PCV (%)	MCV (f1)	MCH (pg)	MCHC (g/dl)
Control	9.66 ± 0.13	10.24 ± 0.14	39.23±1.12	40.61±1.19	10.60±0.41	26.10±0.84
Aflatoxicated sheep.	8.26 ±0.19*	8.80 ± 0.15*	31.40±0.53*	38.14±0.83	10.68±0.16	28.06±0.54

*Significant at P≤ 0.001using T-student test.

Table (3): Mean values ± SE of leucogram (absolute values X 10^3 /µl) of aflatoxicated sheep.

Group	WBCs	Neutrophils	Lymphocytes	Monocytes	Oesinophilis	Basophiles
Control	8.76± 0.18	2.09 ±0.14	6.26± 1.12	0.25± 1.19	0.12± 0.00	0.04± 0.02
Aflatoxicated sheep.	6.02±0.56*	1.92± 0.34	3.74± 0.53	0.21± 0.02	0.10± 0.02	0.04± 0.02

*Significant at P≤ 0.001using T-student test.

Table (4): Liver and kidney function tests of sheep exposed to contaminated ration with aflatoxin B1 (300 ppb).

Biochemical parameters	Sheep exposed to contaminated ration n=50	Sheep exposed to uncontaminated ration n=10
ALT (Iu/ml)	95± 1.6***	31± 1.7
AST (Iu/ml)	92± 1.3***	40± 1.6
AP (U/L)	325± 2.4 ***	139± 1.9
LDH (Iu/l)	2500± 5.6**	730 ± 4.3
Urea (mg/dl)	65± 1.9 **	29± 1.1
Creatin. (mg/dl)	1.88 ±0.03 **	0.9 ± 0.02
T.P (g/dl)	5.1 ±0.02 **	7.5 ± 0.09
Alb. (g/dl)	2.8± 0.01 **	3.8 ± 0.03
Glob (g/dl)	2.3 ±0.02**	3.7 ± 0.02
A/G ratio	1.2 ±0.01	1.02 ± 0.03

** =significant at P ≤ 0.01 ***= significant at P ≤ 0.001

Table (5): Minerals and vitamins provide of affected sheep with aflatoxins B1 (300 ppb).

Minerals and Vitamins	Contaminated ration n=50	Uncontaminated ration n=10
T. Calcium (mg/dl)	7.4 ± 0.3 ***	11.2± 0.92
Inorganic p (mg/dl)	4.1 ± 0.61**	5.7 ± 0.31
S. Magnesium (mg/dl)	1.8 ± 0.03*	3.1 ± 0.11
Sodium (mEq/l)	115 ± 3.2*	135 ± 2.44
Potassium (mEq/l)	2.7 ± 0.12*	3.8 ± 0.09
Vit.A (Iu/dl)	21± 0.94**	48 ± 1.6
Vit. E (ug/dl)	450 ± 3.6**	715 ± 3.9

*Significant at P≤ 0.05 ** =significant at P ≤ 0.01 **= significant at P ≤ 0.001

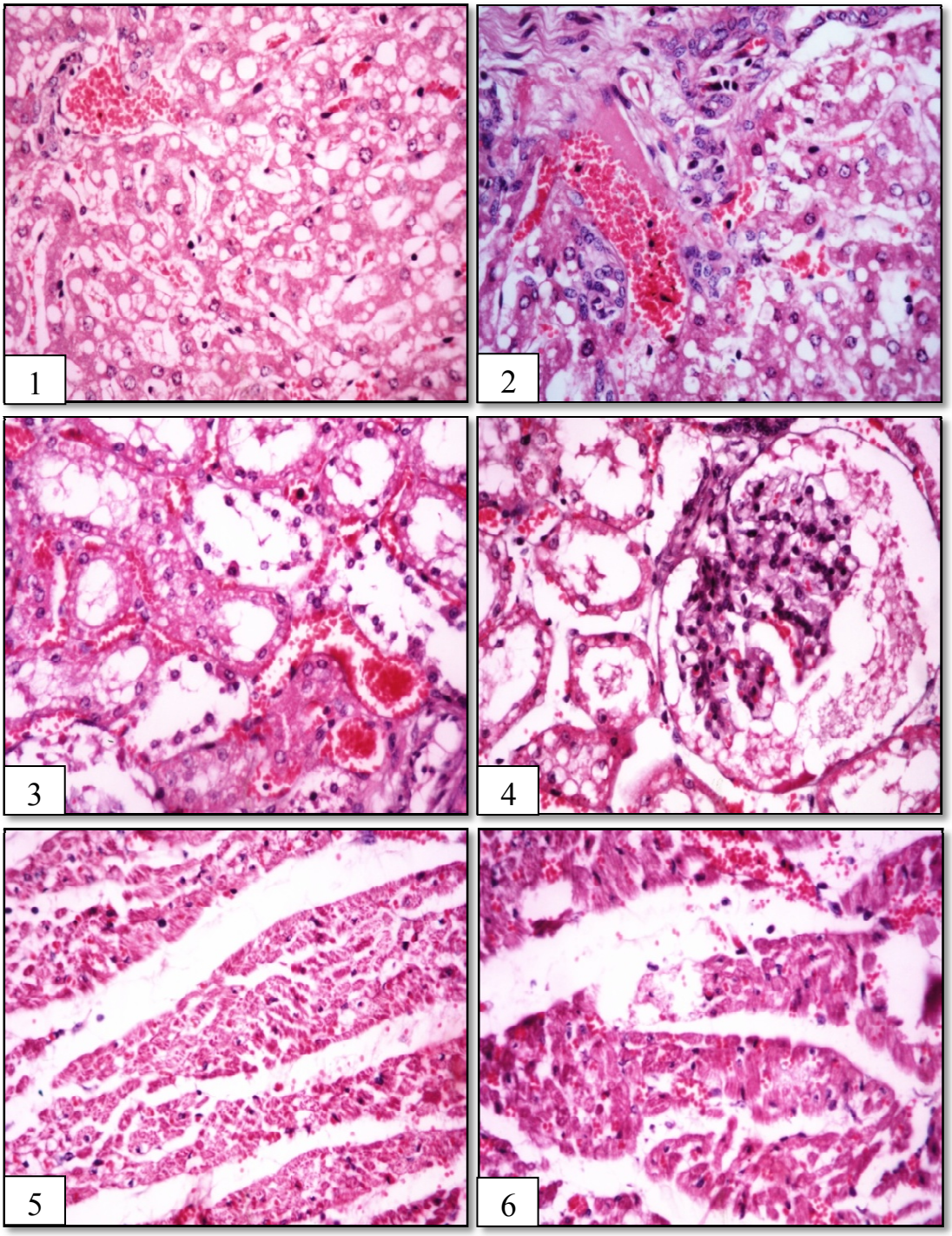

Fig. (1): Liver of sheep fed aflatoxin contaminated feed showing necrosis of hepatocytes and vacuolar degenerative changes in most of them, in addition to disorganization of liver cell arrangement (H & E X 400).

Fig.(2): Liver of sheep fed aflatoxin contaminated feed showing newly formed bile ductules and focal aggregation of vesicular cells distributed in the portal area, as well as congestion of portal blood vessels (H & E X 400).

Fig.(3 and 4): Kidney of sheep fed aflatoxin contaminated feed showing glomerular edema as well as sever edema dispersed the renal tubules, vacuolar degenerative changes of some epithelial lining renal tubules and necrosis of others (H & E X 400).

Fig. (5 and 6): Heart of sheep showing Zenker necrosis of most of cardiac muscles, in addition to severe oedema which dispersed the cardiac muscles and admixed with mononuclear inflammatory cells, associated with hemorrhage in between cardiac muscles (H & E, 200).

Table (6): Residue of aflatoxin in internal organs & muscle in affected and healthy sheep comparing with permissible limits.

Organ	No of sheep=50	Aflatoxin residue Ug/kg	Permissible limit*
Liver	Healthy 15 Affected 35	0 25-50	20 ug/kg
Kidney	Healthy 20 Affected 30	0 45-70	20 ug/kg
Muscle	Healthy 38 Affected 12	0 55-80	20ug/kg

*Permissible limits according to WHO, 1983.

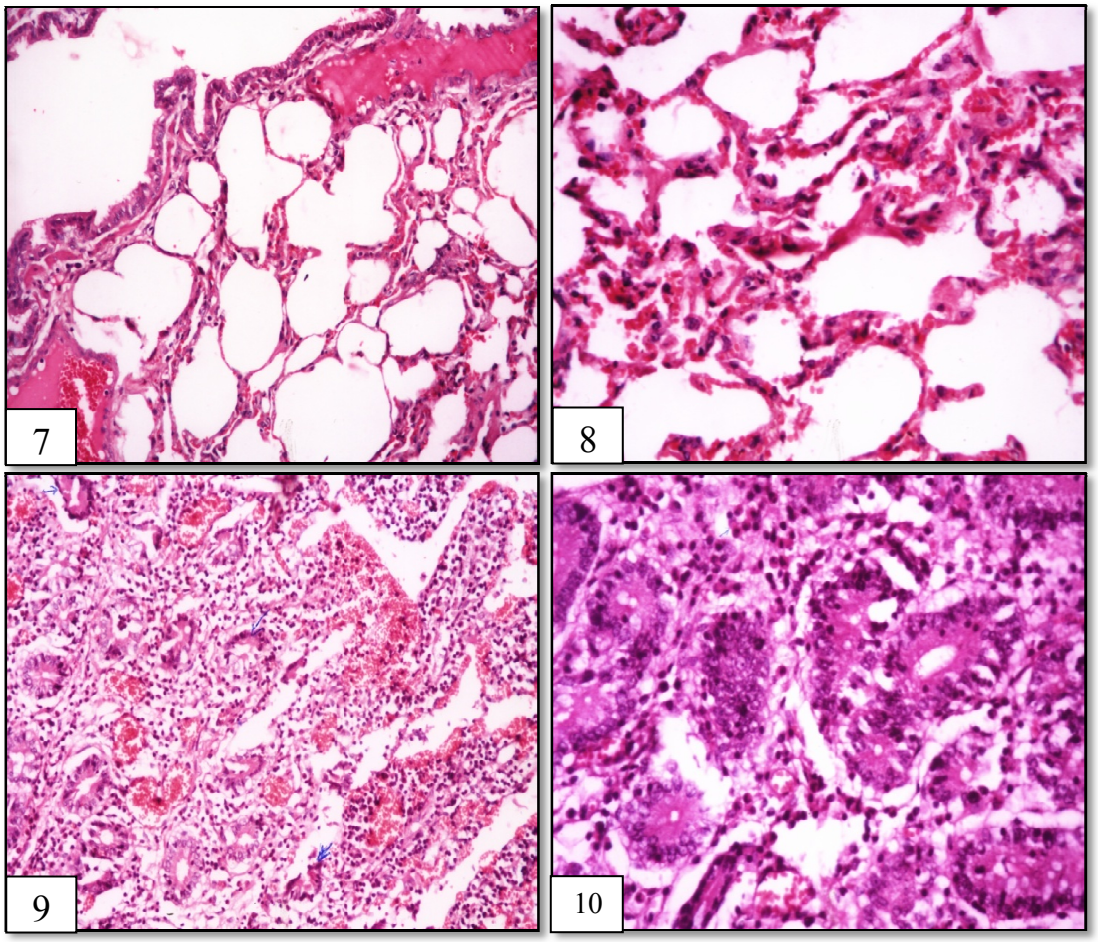

Fig. (7 and 8): Lung of sheep fed aflatoxin contaminated feed showing mild emphysema of some alveoli and dilation of some bronchi with proliferation of the epithelial cells lining some bronchi, associated with congestion of some pulmonary blood vessels and others contain hemolysed blood (H &E X 200 &400).
Fig. (9 and 10): Intestine of sheep showing hemorrhages in the villi and in between the intestinal glands as well as infiltration of mononuclear inflammatory cells associated with necrosis of some intestinal glands (H & E X 100&400).

Discussion

Acute aflatoxicosis, resulting from ingestion of large quantities of aflatoxins, lead to severe hepatic necrosis within hours. Signs referable to liver damage develop rapidly and include icterus, widespread hemorrhage, and elevation of serum hepatic enzymes. Also, aflatoxins bind to nucleic acids and disrupt poly- ribosomes leading to interference with both nucleic acid and protein synthesis. They also result in impaired T cell function (Jones *et al.,* 1996). Al_anati and petzinger, (2006) added to that the inhibition of protein synthesis lead to slow replacement of affected immune cells.

Regards to hematological study, aflatoxin exposed sheep evoked significant decrease in RBCS count, Hb and PCV with normal value of MCV and MCHC indicating presence of normocytic normochromic anemia. Also, leucopenia was detected, that come partially

parallel with the result of Sharma *et al.,* (2011) where reported that AFB1 in mice caused a significant decrease in erythrocytic count, hemoglobin content and lymphocyte count. Significant increase was seen in total leukocytic count and segmented neutropil, that might be due to lower oxygen supply to different tissues, resulting in low energy production. Decrease in RBCS has been attributed to reduction / disturbance in the erythropoisis in bone marrow (Panda *et al.,* 1975) and a faster rate of destruction of peripheral RBCS spleen (Gupta, 1967).

Histopathological findings in this study reveled hemorrhage in between cardiac muscles, hepatocytes, intestinal glands and in the villi, in addition to interstitial hemorrhage in renal tissue. Also, some pulmonary blood vessels contain hemolysed blood. That acute hemorrhage before regeneration occurs with

Uremia explained the normocytic normochromic anemia, which appeared in sheep (Arafa *et al.,* 2006).

Manal *et al.,* (2004) recorded that red blood cells, hemoglobin and packed cell volume were decreased in aflatoxicated goat and may have been occurred due to either blood loss or kidney and liver affection.

Leucopenia appeared in the present investigation may due to destruction or reduction in the rate of formation of leucocytes in bone marrow.

Feldman *et al.,* (2000) recorded that stachybtryotoxicosis has been reported in cattle and sheep and is characterized by progressive and sever leucopenia and thrompocytopenia with focal bone marrow necrosis and significant myeloid hypoplasia. Field cases in sheep had leukocyte counts reported as low as $2.3x10^3$ µl.

In contrast, no significant differences were observed in erythrocyte, leucocytes or differential leukocyte counts in lambs were intoxicated with 2ppm aflatoxin for a period of 37 days (Fernandez *et al.,* 2000).

Iowa state university report (2011) recorded that cattle with 10000 -20000 ppb aflatoxin in feed effect icterus, hemorrhage, liver necrosis and death in one to two weeks. Also, calves (weaning) fed 200 ppb aflatoxin in feed for 2-4 weeks will show reduced weight gain, hemorrhages and possible immune suppression.

According to the present study, the activities of ALT, AST and AP were increased and it has been reported to occur due to altered permeability of hepatocytes (Roger *et al.,* 1991; Edrington *et al.,* 1994) and myocytes (Harrey *et al.,* 1995). Elevation of AP also may be due to hepatic necrosis caused by aflatoxin administration leading to thickening of bile ducts and intrahepatic colestasis (Rager *et al.,* 1991).

In the present study, reduction of albumin values resulted in decrease in A/G ratio. It has been reported that aflatoxin interfere with carbohydrate oxidation, a process which is essential for providing energy needed for protein synthesis (Fernandez *et al.,* 1997). Also, Fernandez *et al.,* (2000) stated that phagocytosis by the neutrophils was higher during the intoxication period and the levels of IGG were elevated.

In our study, microscopical examination of liver revealed that some hepatocytes suffered from necrosis, while the most of them showed vacuolar degenerative changes. In addition to newly formed bile ductules were seen in portal areas. Similar results were reported by Jones *et al.,* (1996), where the most striking and consistent lesion in all species is marked proliferation of small bile ductules at the periphery of hepatic lobules, seen within days of exposure. Changes in hepatocytes include fatty change, swelling and necrosis, although necrosis is not as extensive as in acute exposure. Abou-Rawash *et al.,* (2008) recorded that examination of liver revealed mild signs of toxicity represented by diffuse necrobiotic changes in the form of vacuolation of cytoplasm and vesiculation of nucleus with dispersed chromatin.

Moreover, some vesicular cells with nuclear division were scattered in the portal area as a per-carcinogenic stage appear in our study. Groopman (1994) recorded that epidemiological studies linking aflatoxin exposure to increased incidences of liver cancer.

Values of serum total protein, albumin and globulins were decreased significantly with continues administration of aflatoxins which correlated well with the inhibitory effect of aflatoxin on protein synthesis, and liver damage (Neathery *et al.,* 1980).

Aflatoxins are believed to decrease the availability or quantity of bile salts in the gastrointestinal tract, resulting in decreased absorption of fat soluble vitamins (Cheeke and Shull, 1985).

If vitamin D assimilation is impaired, Ca and P absorption may likewise decrease, which may explain the lowered blood Ca and P concentrations seen in intoxicated sheep in this

study.

Gross findings of dead sheep showed hydrothorax and ascites, so, sudden death of sheep in our investigation may be related to insufficiency of kidney.

In the current study, kidney suffered from glomerulonephrosis, that explain the lowered blood Ca in our study.

Azza and Nadia (2009) indicate increase in manoldialdehyde (index of lipid peroxidation, LPO) in kidneys of aflatoxins-treated rats. Induction of LPO by AFB1 is considered one of the main manifestation of oxidative damage initiated by reactive oxygen species (ROS) and it has been linked with altered membrane structure and enzyme inactivation (Niki *et al.,* 2005). These species trigger cell damage through binding to cell macromolecules as well as membrane, leading to membrane peroxidation which affect the ionic permeability of the membrane and eventually leading to the impairment of cell functioning and cytolysis (Berg *et al.,* 2004). This is supported by another investigation stated that AFB1 is a potent nephrotoxic compound leading to sever degenerative renal damage (Wangikar *et al.,* 2005, Tessari *et al.,* 2006).

Oxidative damage in the cell or tissue occurs when the concentration of ROS generated exceeds the antioxidant capability of the cell (Sies and Stahl, 1995) or when the antioxidant capacity of the cell decreases.

Levels of non-enzymatic antioxidant (vitamin C) and enzymatic antioxidants (glutathione reductase, GR and glucose-6-phosphate dehydrogenase, G-6- PDH) are the main determinants of the antioxidant defense mechanism of the cell, that as detected by Azza and Nadia (2009) in AFB1-treated rats. Their results also showed that aflatoxicosis interfere with the cellular energy supply of rat hearts through its inhibitory action on some markers of energy metabolism indicated by a decrease in glucose and glycogen contents of heart and a reduction in the activities of some glycolytic enzymes, phosphogluco-isomerase (PGI), glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and lactate dehydrogenase (LDH). Indicating aflatoxin B1 induced renal and cardiac damage in rats.

Microscopically, most cardiac muscles were suffered from zenker necrosis. Severe edema were dispersed the muscles.

The obtained results concerned with kidney function tests demonstrated significant increase in serum urea and creatinine values and histopathological alteration of cardiac and renal tissues indicate circulatory insufficiency or nephropathy. Similar findings have been reported in sheep by Suliman *et al.,* (1987); Soliman (1998).

Pulmonary tissue showed proliferation of lining epithelial cells of some bronchi, which may be as defense mechanism against toxin, in addition to hemolysed blood which is evident phenomena in toxin exposed sheep.

In the present investigation, intestinal villi showed hemorrhage and complete necrosis of some intestinal glands were evident, as well as infiltration of mononuclear inflammatory cells, some cases showed infiltration of eosinophils. Evidences of gastritis and enteritis were reported by Pier *et al.,* (1989).

In the present investigation, Aflatoxin B1 induced renal and cardiac damage in sheep. That may be important causes for sudden death.

Histopathological studies revealed that the most affected organs were liver, kidney and heart followed by lung and intestine, which may be attributed to the fact that liver is the organ which responsible for intoxication and kidney responsible for excretion, and the principle cause of death was the affect on functions of vital organs and act on immunosuppressive effect.

In conclusion, it appear that aflatoxicosis in sheep affects the production (mortality, hematological and tissue of internal organs), therefore, the breeding of sheep, as other animals, must be given more care to avoid aflatoxicosis by using healthy feeds and clean environment.

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